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Changes in Terminal Respiratory Pathways of *Bacillus subtilis* During Germination, Outgrowth, and Vegetative Growth

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The chemical and enzymatic properties of the cytochrome system in the particulate preparations obtained from dormant spores, germinated spores, young vegetative cells, and vegetative cells of *Bacillus subtilis* PCI219 were investigated. Difference spectra of particulate fractions from dormant spores of this strain suggested the presence of cytochromes a, a_3, b, $c(+c_1)$, and o. All of the cytochrome components were present in dormant spores and in germinated spores and vegetative cells at all stages which were investigated. Concentrations of cytochromes a, a_3, b, and $c(+c_1)$ increased during germination, outgrowth, and vegetative growth, but that of cytochrome o was highest in dormant spores. As the cytochrome components were reducible by reduced nicotinamide adenine dinucleotide (NADH), they were believed to be metabolically active. Difference spectra of whole-cell suspensions of dormant spores and vegetative cells were coincident with those of the particulate fractions. NADH oxidase and cytochrome c oxidase were present in dormant spores, germinated spores, and vegetative cells at all stages after germination, but succinate cytochrome c reductase was not present in dormant spores. Cytochrome c oxidase and succinate cytochrome c reductase activities increased with growth, but NADH oxidase activity was highest in germinated spores and lowest in vegetative cells. There was no striking difference between the effects of respiratory inhibitors on NADH oxidase in dormant spores and those on NADH oxidase in vegetative cells.

There have been several reports on cytochrome components of vegetative cells of the genus *Bacillus*. It is especially well known that vegetative cells of *B. subtilis* show absorption spectra of cytochrome components which are very similar to those of yeast and mammals; the cytochrome components are cytochromes a, a_3, b, c, and c_1, which are typical respiratory pigments (2, 9, 11). Studies on cytochrome components in vegetative cells of *B. cereus* have been reported by Doi and Halvorson (4) and Schaeffer (10) and those in vegetative cells of *B. megaterium* by Weibull and Bergström (16) and Broberg and Smith (1). However, only a few reports of respiratory pigments of dormant spores of *Bacillus* have been published. The absorption spectrum of cytochrome of *B. subtilis* spores at the temperature of liquid air showed that spores of bacilli differ from their vegetative cells in cytochrome content (7). Doi and Halvorson (4) indicated that cytochromes are lacking in spores of *B. cereus*.

This report confirms the presence of cytochromes in dormant spores of *B. subtilis* and shows quantitative variations of cytochrome components and a few respiratory enzymes that occur at different growth times after germination.

MATERIALS AND METHODS

Bacteria. *B. subtilis* PC1219 and *B. subtilis* JB69 were used in this study. The latter strain, kindly provided by K. Miki, Osaka University, Toyonaka, Osaka, Japan, was a mutant type which was derived from wild-type strain JB15 by irradiation with ultraviolet light.

Dormant spores. Dormant spores were prepared from a 5-day culture of the strain grown on nutrient agar plus 0.01% $MnCl_2$ at 37 C. The harvested spores were washed several times with distilled water and kept frozen at −20 C until used. This preparation contained neither vegetative forms nor debris. When the vegetative form residue was present, spores were freed of vegetative cells by incubating the spore pellet in 20 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 7.2) containing lysozyme at 50 C for 30 min, washed with distilled water, and stored at −20 C.

Germinated spores. Dormant spores preheated at 60 C for 30 min were suspended in 20 mM phosphate buffer (*p*H 7.2) containing L-alanine in a final concentration of 5 mM and incubated at 37 C for 2 hr. Under these conditions, 80 to 90% of the dormant spores underwent germination (as measured by a decrease in the optical density at 650 nm). The germinated spores were collected by centrifugation and washed several times with distilled water.

Young vegetative cells and vegetative cells. Spores germinated by L-alanine were suspended in nutrient broth and incubated at 37 C for 2 hr for young vegetative cells and for 6 hr and 9 hr for vegetative cells. The culture was carried out in 500-ml flasks, each containing 100 ml of the medium, and shaken at a speed of 110 times per min. The collected cells were subsequently treated in the same manner as germinated spores.

Preparation of particulate fraction and soluble fraction from the washed cells. The washed cells of each stage were suspended in 20 mM phosphate buffer (*p*H 7.5). About 3 ml of the thick dormant spore suspension, together with 20 g of glass beads, was treated in a 10-kc sonic oscillator at 2 to 3 C for 40 min; the germinated spore suspension was treated for 20 min under the same conditions. Young vegetative cell and vegetative cell suspensions were oscillated for 10 min without glass beads. After the treatment, the broken cell suspension was centrifuged at 10,500 × *g* for 40 min at 4 C, and the supernatant fluid of the cell extracts was again centrifuged at 135,500 × *g* for 3 hr and separated into particulate and soluble fractions. The precipitate thus obtained was suspended in 20 mM phosphate buffer (*p*H 7.5) and centrifuged again at 135,500 × *g* for 1 hr. The precipitate collected by centrifugation was dissolved in a minimal volume of the same buffer and stored at −20 C.

Measurement of the difference spectra. Measurements of the difference spectra between the reduced preparation and the oxidized preparation were performed with a recording spectrophotometer (Cary, model 14) supplied with 0 to 0.1 and 0 to 1.0 absorbance slide wires, by using a 1-cm light path cuvette, at 25 C; at the temperature of liquid nitrogen, a 0.2-cm light path cuvette was employed. For measurements of the difference spectra of preparations from dormant spores, germinated spores, and young vegetative cells, and also for the determination of the effect of CO on absorption spectra, a modified Thunberg-type cuvette was used. The difference spectra between CO-treated reduced preparation and untreated reduced preparation were also measured with a recording spectrophotometer (Cary, model 14). A gentle stream of CO was added to the anaerobic sample for 1 min.

The concentration of each cytochrome in the particulate fractions was determined from the reduced nicotinamide adenine dinucleotide (NADH) reduced-minus-oxidized difference spectra and CO reduced-minus-reduced difference spectra. The following wavelength pairs and millimolar extinction coefficients were used for the determination of the components: cytochrome $a(+a_3)$, ϵ(604–623 nm) = 16 mM^{-1} cm^{-1}; cytochrome b, ϵ(562–574 nm) = 20 mM^{-1} cm^{-1}; cytochrome $c(+c_1)$, ϵ(552–540 nm) = 19 mM^{-1} cm^{-1}; cytochrome $a_3(+a)$, ϵ(444–455 nm) = 91 mM^{-1} cm^{-1} for reduced-minus-oxidized difference spectra and cytochrome a_3, ϵ(430–445 nm) = 82 mM^{-1} cm^{-1}; cytochrome o, ϵ(415–480 nm) = 110 mM^{-1} cm^{-1} for CO reduced-minus-reduced difference spectra. The lipase treatment of particulate fraction from vegetative cells was carried out according to the method of Broberg and Smith (1).

Measurement of each enzyme activity. NADH oxidase [EC 1.6.99.3, reduced-NAD:(acceptor)oxidoreductase] activity was determined spectrophotometrically by the decrease in absorbance at 340 nm of NADH. The reaction mixture for NADH oxidase activity contained 150 μmoles of phosphate buffer (*p*H 7.5), 0.67 μmole of NADH, enzyme plus additions as indicated, and water to 3.0 ml. The specific activity was expressed as nanomoles of NADH oxidized per minute per milligram of protein.

Succinate cytochrome *c* reductase [EC 1.3.99.1, succinate:(acceptor)oxidoreductase] activity was measured by the increase in optical density at 550 nm of cytochrome *c* in the presence of cyanide. The cuvette contained 150 μmoles of phosphate buffer (*p*H 7.5), 15 μmoles of succinate, 3 μmoles of KCN, 150 nmoles of cytochrome *c*, enzyme, and water to 3.0 ml. The specific activity was expressed as nanomoles of cytochrome *c* reduced per minute per milligram of protein.

Cytochrome *c* oxidase (EC 1.9.3.1, ferrocytochrome *c*:oxygen oxidoreductase) activity was measured by following the decrease in optical density at 550 nm of chemically reduced cytochrome *c*. The reaction mixture contained 150 μmoles of phosphate buffer (*p*H 6.0), approximately 75 nmoles of ferrocytochrome *c*, enzyme, and water to 3.0 ml. Ferrocytochrome *c* was obtained by reducing cytochrome *c* with a small amount of ascorbic acid and treating the reduced cytochrome *c* with a Sephadex G-25 column to remove the reductant and the oxidized products of ascorbate. The specific activity was calculated from the first-order velocity by the method of Smith and Conrad (13) and was expressed as κ per second per milligram of protein. Each enzyme activity was measured at 25 C with a recording spectrophotometer (Cary, model 14) and a cuvette of 1-cm light path, except for measurements of succinate cytochrome *c* reductase and cytochrome *c* oxidase activities from dormant spores, germinated spores, and young vegetative cells. In these cases a dual-wavelength spectrophotometer (Aminco-Chance) was used. Protein was determined by the method of Lowry et al. (8).

Chemicals. NADH was purchased from Sigma Chemical Co. Crystalline cytochrome *c*, prepared from *Candida krusei*, was purchased from Sankyo Co., and piericidin A was a gift of S. Tamura, Faculty of Agriculture, University of Tokyo. Antimycin A was obtained from Seikagaku Kogyo Co., amytal (sodium amobarbital) from Nippon Shinyaku Co., alcohol dehydrogenase from Boehringer Mannheim Co., pancreatic lipase from Sigma Chemical Co., and lysozyme from Kyowa Fermentation Industry Co. All other chemicals were of the finest grade.

Inhibitors such as dicumarol and sodium amytal were suspended in water, and 3 N KOH or 1 N HCl was added dropwise until a clear solution was obtained.

The solution was adjusted to *p*H 7.5 and brought to the suitable concentrations. Relatively insoluble compounds such as rotenone, piericidin A, and antimycin A were dissolved in a small amount of methanol and then diluted with distilled water. *o*-Phenanthroline was dissolved in a small amount of acetone.

RESULTS

Cytochrome components in the particulate fractions from each type of cell of B. subtilis PCI219. NADH reduced-minus-oxidized difference spectra and CO difference spectra of particulate fractions from dormant spores, germinated spores, young vegetative cells, and vegetative cells of *B. subtilis* PCI219 are shown in Fig. 1, and the cytochrome components obtained from Fig. 1 are arranged in tabular form (Table 1). All types of cells except dormant spores contained cytochromes a, a_3, b, $c(+c_1)$, and o. Dormant spores seemed to contain at least cytochromes b, $c(+c_1)$ and o; peaks indicative of cytochromes a and a_3 were not apparent.

The concentrations of individual cytochrome in each type of cell were determined from the above two kinds of difference spectra. As shown in Fig. 2, cytochromes a, a_3, and b increased in parallel with each other (except that dormant spores seemed not to contain cytochromes a and a_3), but cytochrome $c(+c_1)$ increased independently. The concentration of cytochrome o was highest in dormant spores; germinated spores, young vegetative cells, and vegetative cells had half as much cytochrome o as dormant spores. The differences between the concentrations of cytochrome a_3 obtained from NADH reduced-minus-oxidized difference spectra and those of a_3 obtained from CO difference spectra may be dependent on the influence of cytochrome o.

The absorption spectrum of the dormant spore particles at the temperature of liquid nitrogen was measured to discover whether cytochromes a and a_3 were present in the dormant spores. Absorption bands which appeared at 601 and 440 nm (Fig. 3) suggested that dormant spores of this strain contain a small quantity of cytochromes a and a_3.

These experimental results showed that the dormant spores of *B. subtilis* PCI219 contain cytochromes a, a_3, b, $c(+c_1)$, and o, and the concentration of each cytochrome except cytochrome o increases during germination, outgrowth, and vegetative growth.

Lipase treatment of particulate fraction from vegetative cells (9-hr culture) of B. subtilis PCI219. To ascertain the presence of the two CO-binding pigments, the particulate fraction from vegetative cells was treated with pancreatic lipase by the method of Broberg and Smith (1). A suspension of particulate fraction which had been incubated with lipase was centrifuged at 33,000 × *g* for 1 hr and separated into supernatant and residue fractions.

The $Na_2S_2O_4$ reduced-minus-oxidized difference spectrum of the lipase-treated residue (Fig. 4) indicated a prominent cytochrome a 602-nm peak and a cytochrome b 564-nm peak in the visible region, and a cytochrome a_3 442-nm shoulder and a cytochrome b 434-nm peak in the Soret region. However, almost all of the cytochrome $c(+c_1)$ in the untreated particulate fraction was absent in both the visible region and the Soret region. The CO difference spectrum of the residue indicated only one CO-binding pigment with the characteristics of cytochrome a_3; it appeared that cytochrome o had been specifically removed from the particulate fraction by the lipase treatment.

A $Na_2S_2O_4$ reduced-minus-oxidized difference spectrum of the supernatant fraction from the lipase incubation indicated maxima at 552 and 422 nm, which are indicative of cytochrome c. A CO difference spectrum of the same material had a peak at 413 nm and a trough at 430 nm, which are indicative of cytochrome o. Thus, the properties of the two fractions support the conclusion that the a_3 pigment has remained associated with the residue, while the o pigment is no longer precipitable after the lipase treatment. From the experimental results, the existence of cytochrome o was confirmed.

Difference spectra of whole cells of dormant spores and vegetative cells (9-hr culture) of B. subtilis PCI219. To settle the problem of whether cytochrome o was a natural constituent of *B. subtilis* PCI219 cells or an artifact due to the procedures used in preparation of the particulate fractions, difference spectra were also run on a suspension of whole dormant spores in comparison with that of vegetative cells. As can be seen in Fig. 5, the difference spectra pattern for dormant spores reduced with $Na_2S_2O_4$ is similar to the pattern for the particulate fraction (Fig. 1a), and the pattern for vegetative cells reduced with succinate is similar to the pattern for particulate preparations reduced with NADH (Fig. 1e). Therefore, it is believed that cytochrome o in *B. subtilis* PCI219 is a natural constituent.

Difference spectra of particulate fractions from dormant spores and vegetative cells (9-hr culture) of B. subtilis JB69. There are many reports for cytochromes of *B. subtilis* (2, 9, 11), but none on the presence of cytochrome o. This paper reports evidence that the *B. subtilis* PCI 219 strain used in this study contains cytochrome o as well as a_3; but on the other hand, it gives rise to a question concerning a historical transition from cyto-

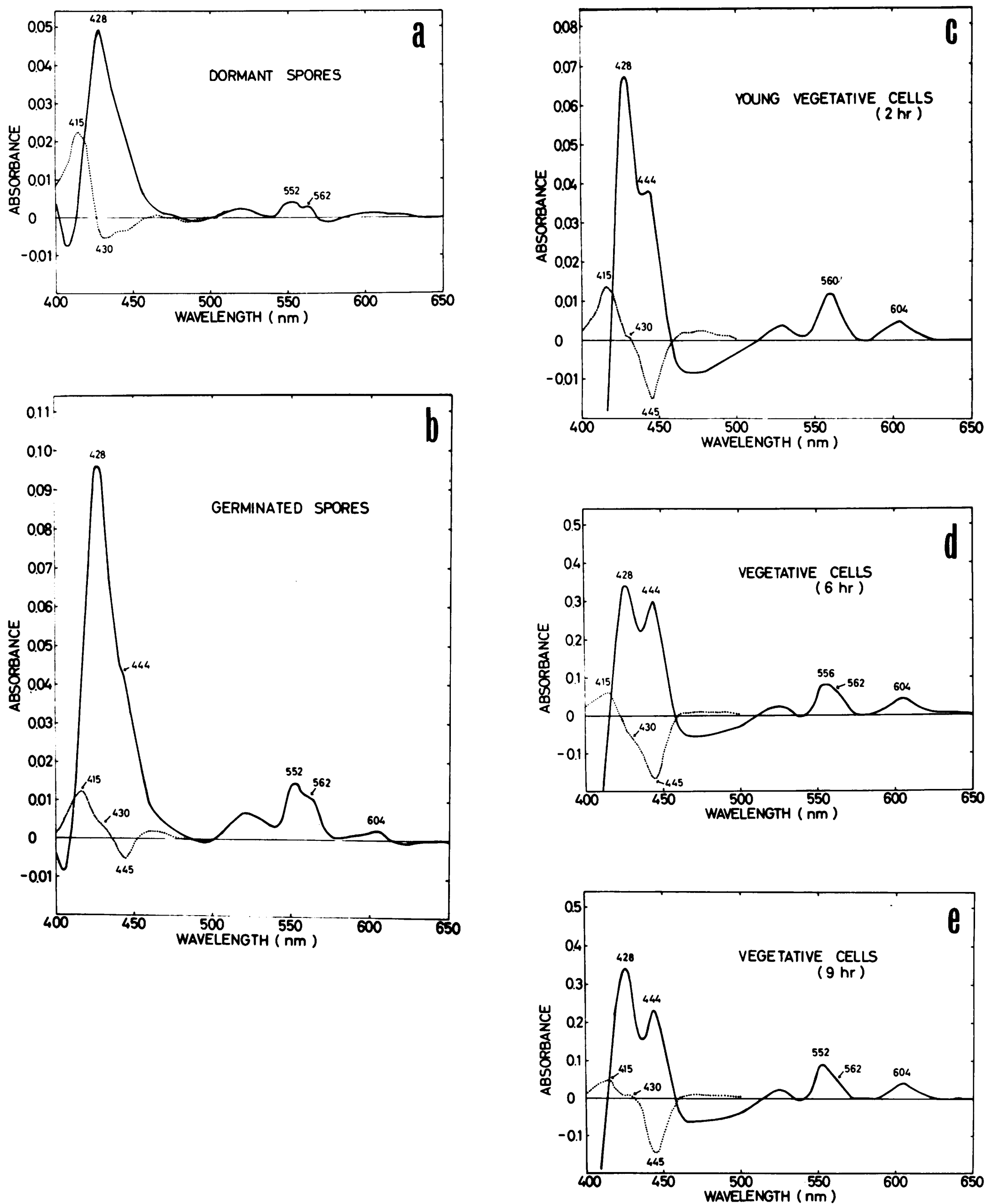

FIG. 1. *Difference spectra of particulate fractions from dormant spores, germinated spores, young vegetative cells, and vegetative cells of B. subtilis PCI219. Reaction mixture consisted of 150 μmoles of phosphate buffer (pH 7.5), washed particles (5 to 20 mg of protein), and water to a final volume of 3.0 ml. Assay system was reduced by adding 2 μmoles of NADH, 105 μmoles of ethyl alcohol, and 150 units of alcohol dehydrogenase. Reaction was carried out at 25 C in a 1-cm cuvette. CO was bubbled through reduced samples for 1 min. Solid lines indicate reduced-minus-oxidized difference spectra; dotted lines indicate CO reduced-minus-reduced difference spectra.*

TABLE 1. *Spectral changes and cytochrome components on the particulate fractions from each type of cell of B. subtilis PCI219*[a]

Source	Reduced-minus-oxidized difference spectra[b]	CO reduced-minus-reduced difference spectra[b]	Cytochromes
Dormant spores	428↑ 552↑ 562↑	415↑ 430↓	$b,c(+c_1),o$
Germinated spores	428↑ 444* 552↑ 562↑ 604↑	415↑ 430* 445↓	$a,a_3,b,c(+c_1),o$
Young vegetative cells	428↑ 444↑ 560↑ 604↑	415↑ 430* 445↓	$a,a_3,b,c(+c_1),o$
Vegetative cells (6 hr)	428↑ 444↑ 556↑ 562* 604↑	415↑ 430* 445↓	$a,a_3,b,c(+c_1),o$
Vegetative cells (9 hr)	428↑ 444↑ 552↑ 562* 604↑	415↑ 430* 445↓	$a,a_3,b,c(+c_1),o$

[a] Tabulated from the data in Fig. 1.
[b] ↑, Maximum wavelength of peak; ↓, minimum wavelength of trough; *, wavelength of clear shoulder.

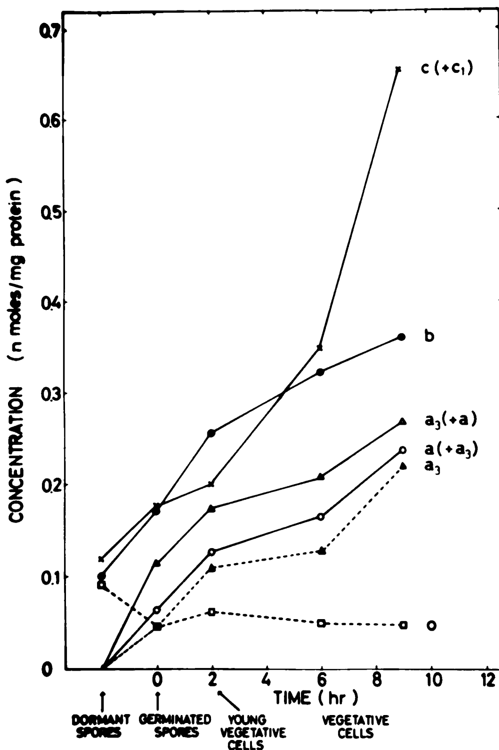

FIG. 2. *Concentration of individual cytochrome in particulate fractions from dormant spores, germinated spores, young vegetative cells, and vegetative cells of B. subtilis PCI219. Each value represents the average of at least five determinations. Solid lines indicate values from reduced-minus-oxidized difference spectra; dotted lines indicate those from CO reduced-minus-reduced difference spectra.*

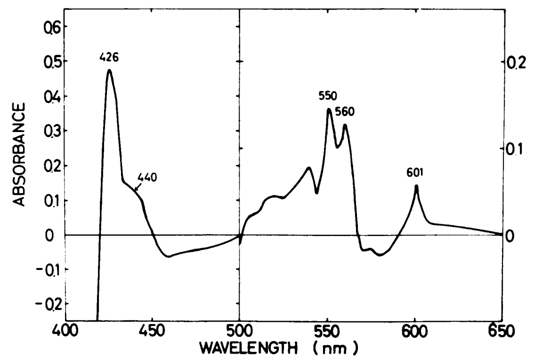

FIG. 3. *Reduced-minus-oxidized difference spectrum of particulate fraction from dormant spores of B. subtilis PCI219 at the temperature of liquid nitrogen. Assay system contained 2.5 mg of washed particles in 0.8 ml of 50 mM phosphate buffer (pH 7.5) containing 50% by volume of glycerol. Assay system was reduced by the addition of a small amount of sodium sulfide in a 0.2-cm cuvette at room temperature and brought to 77 K.*

chrome *o* to a_3 during germination. Consequently, the difference spectra of dormant spores of *B. subtilis* JB69, which has been known to contain cytochrome a_3 as its only CO-binding respiratory pigment in vegetative cells (9), were examined (Fig. 6). The cytochrome components and the absorption peaks of the reduced form obtained from dormant spores were as follows: 604 and 444 nm for cytochromes *a* and a_3; 562 nm for cytochrome *b*; and 552 nm for cytochrome $c(+c_1)$. Absorption bands of CO difference spectra of dormant spores revealed a peak at 424 nm and a trough at 445 nm. This fact indicated that dormant spores of *B. subtilis* JB69 contain only cytochrome a_3, unlike the spores of *B. subtilis* PCI219. Difference spectra of vegetative cells of this strain (Fig. 6b) were almost similar to those of dormant spores. It was clear from the result that a transition from cytochrome *o* to a_3 does not occur during germination.

Comparison of activities of several electron transport enzymes in particulate fractions from each type of cell of B. subtilis PCI219. As shown in Fig. 7, dormant spores have a relatively high level of particulate NADH oxidase activity, and the germinated spores and young vegetative cells have four to five times as high a level of the enzyme activity as the dormant spores, but NADH

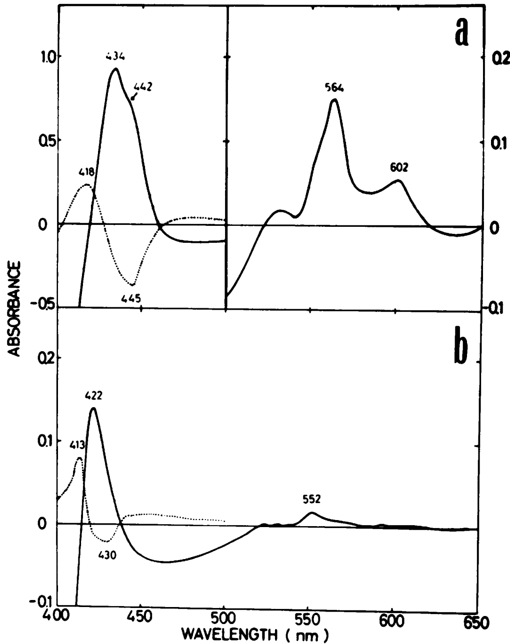

FIG. 4. *Effect of pancreatic lipase on particulate fraction from vegetative cells (9-hr culture) of B. subtilis PCI219. $Na_2S_2O_4$ reduced-minus-oxidized difference spectra and CO reduced-minus-reduced difference spectra of residue (a) and supernatant (b) fractions from incubation of particulate fraction with pancreatic lipase. Incubation was carried out at 37 C for 7 hr, followed by centrifugation at 33,000 × g for 1 hr. CO was bubbled through reduced samples for 3 min. Solid lines indicate reduced-minus-oxidized difference spectra; dotted lines indicate CO reduced-minus-reduced difference spectra.*

oxidase activity in vegetative cells was found to be lower than that in dormant spores. On the other hand, particulate succinate cytochrome *c* reductase was absent in dormant spores, but the enzyme activity appeared slightly in germinated spores and increased remarkably with vegetative growth. A very low level of cytochrome *c* oxidase activity in dormant spores is in agreement with the presence of the cytochrome system. However, the fact that cytochrome *c* oxidase activity in germinated spores and young vegetative cells is almost like that in dormant spores is in contrast with the increase of cytochrome a_3 during germination and outgrowth. It is interesting that, although the increase of cytochrome *c* oxidase and succinate cytochrome *c* reductase activities showed almost the same pattern, NADH oxidase activity showed a fairly different pattern from these two enzymes. The soluble NADH oxidase activities were approximately the same throughout each stage.

Effect of various inhibitors on NADH oxidase system in particulate fractions from dormant spores and vegetative cells (9-hr culture) of B. subtilis PCI219. As shown in Table 2, the presence of 5 mM amytal and 0.025 mM rotenone caused little inhibition of NADH oxidase in particulate fractions of both cells. On the other hand, 96% of the enzyme activity in dormant spores and 74% of that in vegetative cells was inhibited by 0.025 mM piericidin A, and its inhibitory effects declined with decreasing concentration as shown. In dormant spores, 0.045 mM antimycin A inhibited 61% of NADH oxidase ac-

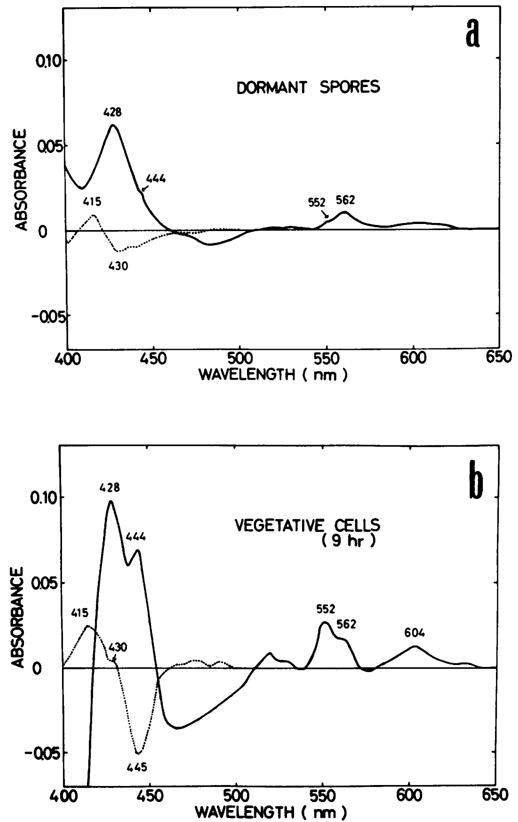

FIG. 5. *Difference spectra of the whole cells of dormant spores and vegetative cells (9-hr culture) of B. subtilis PCI219. Assay system contained a proper quantity of whole cell suspension, 150 μmoles of phosphate buffer (pH 7.5), and water to a final volume of 3.0 ml. Assay system was reduced by adding a small amount of sodium sulfide for dormant spores and 30 μmoles of succinate for vegetative cells. Reaction was carried out at 25 C in a 1-cm cuvette. Reference cuvette was kept in the oxidized state with bubbling oxygen for 5 min before scanning. CO was bubbled through reduced samples for 1 min. Solid lines indicate reduced-minus-oxidized difference spectra; dotted lines indicate CO reduced-minus-reduced difference spectra.*

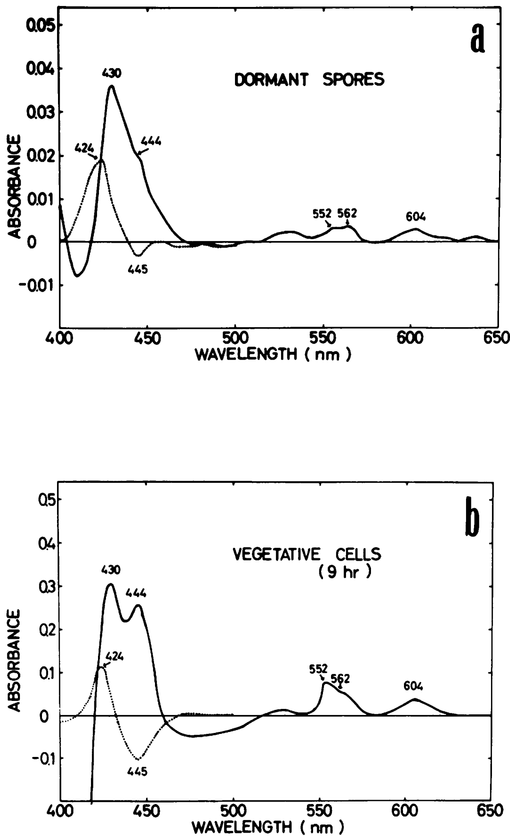

FIG. 6. *Difference spectra of particulate fractions from dormant spores and vegetative cells (9-hr culture) of B. subtilis JB69. Assay mixture and procedure of difference spectra as in Fig. 1.*

tivity, and in vegetative cells, 41%. When 1 mM potassium cyanide was added, there was 64% inhibition in the former and 50% in the latter. However, NADH oxidases in both cells were almost insensitive to 1 mM sodium azide. In addition, the possibility of finding the inhibitory effect on these enzymes of a variety of chelating agents has been investigated. Of the chelating agents examined, 5 mM *o*-phenanthroline inhibited 49% of NADH oxidase in dormant spores and 47% of that in vegetative cells, but the chelating agents other than *o*-phenanthroline gave little inhibition. Moreover, 0.2 mM dicumarol inhibited 50 to 63% of these enzymes, and the sulfhydryl binding agent was also inhibitory.

Comparison of effect of respiratory inhibitors on NADH oxidase activities in particulate fractions from dormant spores, germinated spores, and vegetative cells (9-hr culture) of B. subtilis PCI219. The effect of three respiratory inhibitors (with different action sites on the electron transfer pathway of the NADH oxidase system) on NADH oxidase activity in germinated spores was compared with the effect of the three inhibitors on NADH oxidase activities in dormant spores and vegetative cells (Table 3). Amytal (10 mM) showed little inhibition of NADH oxidase in particulate fractions from three kinds of cells, but 0.045 mM antimycin A inhibited 61 to 63% of NADH oxidase activities in dormant spores and germinated spores and 41% of that in vegetative cells. On the other hand, 1 mM cyanide inhibited 50 to 64% of the enzyme activities in dormant spores and vegetative cells and 28% of that in germinated spores. From the facts that (i) although NADH oxidase activity in germinated spores is very high, only 28% is inhibited by cyanide, and (ii) although concentration of cytochrome a_3 in germinated spores is higher than that in dormant spores, cytochrome *c* oxidase activity in the former is almost the same as in the latter, it seems likely that the cytochrome system in germinated spores is incomplete. NADH oxidase activity of another system, which is not concerned with cytochrome *b*, may therefore increase in germinated spores. This idea may be connected with NADH oxidase activity in young vegetative cells.

Comparison of particulate NADH oxidase and cytochrome c oxidase activities between vegetative cells (9-hr culture) of B. subtilis PCI219 and those of B. subtilis JB69. To determine the influence of cytochrome *o* on NADH oxidase and cytochrome *c* oxidase activities, activities of par-

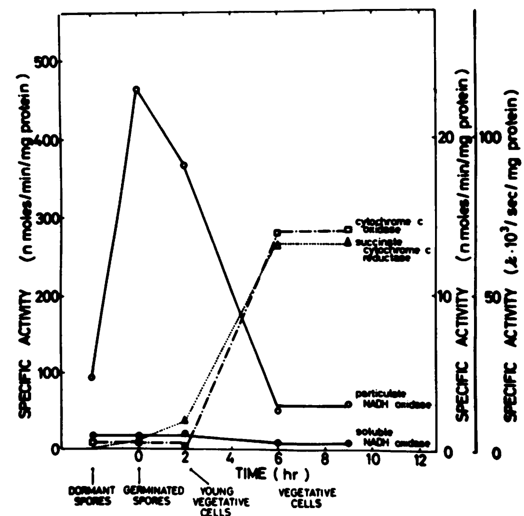

FIG. 7. *Activities of particulate NADH oxidase, succinate cytochrome c reductase, and cytochrome c oxidase in dormant spores, germinated spores, young vegetative cells, and vegetative cells of B. subtilis PCI219. Assay systems and determination of specific activities as in Materials and Methods.*

TABLE 2. *Effect of various inhibitors on NADH oxidase activities of particulate fractions from dormant spores and vegetative cells (9-hr culture) of B. subtilis PCI219*

Inhibitors[a]	Concn (M)	Inhibition (%)	
		Dormant spores	Vegetative cells
Amytal	5×10^{-3}	12	11
Rotenone	2.5×10^{-5}	22	9
Piericidin A	2.5×10^{-5}	96	74
	10^{-5}	81	45
	5×10^{-6}	58	25
Antimycin A	4.5×10^{-5}	61	41
	9×10^{-6}	33	0
BAL	5×10^{-3}	8	7
KCN	10^{-3}	64	50
	10^{-4}	26	16
NaN_3	10^{-3}	19	0
EDTA	10^{-3}	0	0
Allyl-thiourea	10^{-3}	0	0
Tiron	10^{-2}	0	9
α-α'-Dipyridyl	10^{-3}	0	8
o-Phenanthroline	5×10^{-3}	49	47
	5×10^{-4}	21	7
Dicumarol	2×10^{-4}	50	63
	5×10^{-5}	19	22
PCMS	2×10^{-3}	86	90
	5×10^{-4}	35	57

[a] Abbreviations: BAL, 2,3-dimercaptopropanol; EDTA, ethylenediaminetetraacetic acid; PCMS, *p*-chloromercuribenzenesulfonate.

ticulate fractions from vegetative cells of *B. subtilis* PCI219 were measured in comparison with those from *B. subtilis* JB69 (Table 4). In spite of the existence of cytochrome *o*, the enzyme activities were almost the same in the dormant spores of both strains and in the vegetative cells of the two strains used. Moreover, the effect of potassium cyanide on particulate NADH oxidase and cytochrome *c* oxidase activities from vegetative cells of the two strains was investigated. Both cytochrome *c* oxidase activities were completely inhibited, but neither NADH oxidase activity was completely inhibited by 2 mM cyanide (Fig. 8). The effect of cyanide on NADH oxidase activity was much less than that of cyanide on cytochrome *c* oxidase activity, and it was difficult to obtain the complete inhibition of NADH oxidase activity with cyanide.

TABLE 3. *Comparison of effect of amytal, antimycin A, and KCN on NADH oxidase activities of particulate fractions from dormant spores, germinated spores, and vegetative cells (9-hr culture) of B. subtilis PCI219*

Inhibitors	Concn (M)	Inhibition (%)		
		Dormant spores	Germinated spores	Vegetative cells
Amytal	10^{-2}	7	5	2
Antimycin A	4.5×10^{-5}	61	63	41
	1.8×10^{-5}	54	40	22
	9×10^{-6}	33	22	0
	3×10^{-6}	0	19	—
KCN	10^{-3}	64	28	50
	5×10^{-4}	44	16	30
	10^{-4}	26	0	16

TABLE 4. *Comparison of activities of particulate NADH oxidase and cytochrome c oxidase from dormant spores and vegetative cells (9-hr culture) of B. subtilis PCI219 and B. subtilis JB69*

Enzyme	*B. subtilis* PCI219		*B. subtilis* JB69	
	Dormant spores	Vegetative cells	Dormant spores	Vegetative cells
NADH oxidase[a]	94	59	107	67
Cytochrome *c* oxidase[b]	3	71	3	76

[a] Expressed as nanomoles per minute per milligram of protein.

[b] Expressed as $\kappa \cdot 10^3$ per minute per milligram of protein.

From these results it is reasonable to suggest that cytochrome *o* is not essential for oxidation of NADH, and the NADH oxidase system includes both cytochrome *c* oxidase and another oxidase as its terminal oxidase system.

DISCUSSION

I have shown that dormant spores have a full component of cytochromes [a, a_3, b, $c(+c_1)$ and o] as electron-carrying particles, which are very similar to those of vegetative cells. In addition, concentrations of main cytochrome components and activity of succinate cytochrome *c* reductase increased during germination, outgrowth, and vegetative growth. Particulate NADH oxidase activity was highest in germinated spores,

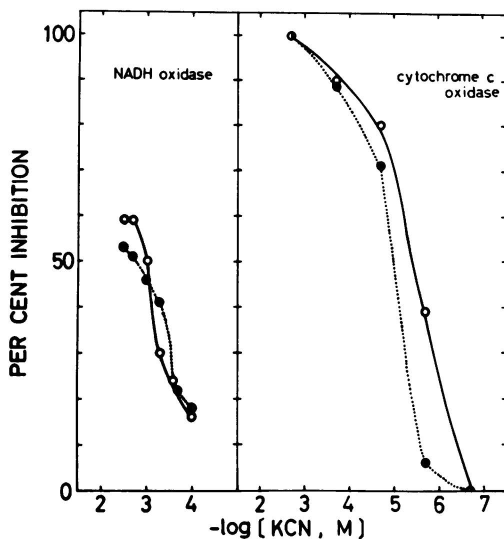

FIG. 8. *Effect of KCN on particulate NADH oxidase and cytochrome c oxidase in vegetative cells (9-hr culture) of B. subtilis PCI219* (O) *and B. subtilis JB69* (●).

and cytochrome *c* oxidase activity increased during vegetative growth but not during germination and outgrowth. There was little or no difference between the main electron pathways of dormant spores and those of vegetative cells.

The presence of cytochromes in dormant spores agrees with Keilin and Hartree (7), who showed the presence of cytochromes in *B. subtilis* spores by observation of difference spectra at low temperature of liquid air. However, Doi and Halvorson (4) reported that cytochromes are lacking in *B. cereus* spores. The discrepancy between that report and the present one may be due to differences both in species and culture conditions in which dormant spores were formed.

The *B. subtilis* strain used in this study is capable of synthesizing two CO-binding pigments, cytochromes *o* and a_3, as cytochrome *c* oxidase. The two components were separated by treatment of particulate fraction from vegetative cells with an extract of pancreatic lipase; the cytochrome a_3 remained with lipase-treated residue, but the cytochrome *o* was removed by the treatment. This result was the same as that reported by Broberg and Smith (1). Dormant spores of *B. subtilis* JB69, which contains cytochrome a_3 as its only CO-binding respiratory pigment in vegetative cells, showed only the presence of cytochrome a_3.

It has been shown by CO difference spectra or photochemical action spectra that some microorganisms contain either cytochrome *o* or both cytochromes a_3 and *o* as their functional oxidase (3, 5, 6, 12, 14, 15). With regard to organisms belonging to the genus *Bacillus*, Broberg and Smith (1) found, on the basis of CO difference spectra, that *B. megaterium* KM contains significant amounts of both cytochromes *o* and a_3.

The physiological significance of cytochrome *o* in dormant spores and other cells is unknown as yet. Broberg and Smith (1) have stated that cytochrome *o* is not essential for oxidation of NADH in *B. megaterium* KM. The results obtained from my experiments are very much in agreement.

The absence of succinate cytochrome *c* reductase in the dormant spore particles is in agreement with the results reported by Doi and Halvorson (4). However, relatively high particulate NADH oxidase activity and very low soluble NADH oxidase activity in dormant spores are in contrast with their results. Judging from these results the functional electron transport system in dormant spores of *B. subtilis* appears to be the particulate NADH oxidase system.

As shown in Table 2, the effects of various inhibitors on the particulate NADH oxidases are almost the same in dormant spores as in vegetative cells. The fact that 39 to 59% of the NADH oxidase activity from both cells is antimycin A-insensitive and 36 to 50% of the enzyme activity is cyanide-insensitive coincides with the idea of Miki et al (9). This idea is that, in the particles from *B. subtilis*, electrons are transferred from the substrate to oxygen considerably more rapidly through the pathway which does not include type *b* component, that is, an electron transfer pathway such as in flavine respiration. This pathway seems to play an important role in germinated spores, as they show very high particulate NADH oxidase activity and a decrease of sensitivity of the enzyme to cyanide. This problem is being actively studied at present.

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